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Zidovudine-Resistant Human Immunodeficiency Virus Selected by Passage in Cell Culture

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Variants of human immunodeficiency virus (HIV) with reduced sensitivity to zidovudine (3′-azido-3′-deoxythymidine) have been selected by passage of virus in cell culture in the presence of drug. Wild-type, sensitive virus became partially resistant to zidovudine by passage 12 (50% inhibitory dose values measured in HeLa CD4$^+$ cells increased from 0.014 to 0.2 μM), and genetic analysis using the polymerase chain reaction revealed that mutations in the reverse transcriptase coding region identical to those seen in clinical isolates from treated individuals had occurred. The order of appearance of these resistance mutations in passaged virus was also similar to that in clinical isolates. The partially resistant strain, HIVRTMC/F, became highly zidovudine resistant by passage 12 (50% inhibitory dose values increased from 0.4 to 2.5 μM during passages 7 to 11). Nucleotide sequence analysis of the reverse transcriptase from this variant revealed a novel amino acid substitution (Lys→Glu) at codon 219. A different substitution at this codon (Lys→Gln) had been seen previously in clinical isolates. When this mutation was created in HIVRTMC/F by site-directed mutagenesis, the resulting partially resistant virus became highly resistant, thus confirming the significance of this change. In view of the possibility that this mutation might occur in HIV isolates during treatment of patients, we adapted our selective polymerase chain reaction procedure to enable screening for this change in clinical samples. The virus passage procedure described here may be useful for gaining further insight into the mutational events occurring during the development of resistance to zidovudine and other HIV inhibitors.

The potential development of drug resistance is a common concern during the clinical use of antiviral agents, although our understanding of resistance to many antiviral drugs has come from studying virus mutants selected in cell culture (for a review, see reference 5). Subsequent investigation of virus isolated from treated individuals has largely substantiated predictions based on laboratory studies as to the likely nature and frequency of resistant virus occurring during treatment (5). Unfortunately, this approach has not proved useful in the study of human immunodeficiency virus (HIV) resistance to zidovudine (3′-azido-3′-deoxythymidine), as attempts to select resistant strains in cell culture have so far been unsuccessful (15, 21). However, analysis of HIV obtained from treated individuals revealed that resistance frequently occurs during prolonged use of zidovudine (12). Isolates may initially become partially resistant at early treatment times and develop increasing resistance as treatment continues (3, 18). Studies on relatively small patient groups indicate that partially resistant isolates emerge more rapidly when HIV infection is advanced than when treatment is begun during earlier-stage infection (3, 12, 18). Highly resistant variants have so far been isolated only from treated individuals with late-stage disease (12).

Resistance is due to mutation in the HIV reverse transcriptase (RT), the target of the triphosphate form of zidovudine (14). Comparative nucleotide sequence analysis of RT cloned from clinical isolates identified predicted substitutions at four specific amino acid residues associated with resistance (14). HIV variants were constructed by site-directed mutagenesis with combinations of these mutations in the RT coding region within an infectious molecular clone (HXB2-D) (14). Virus with all four amino acid changes in RT was highly resistant to zidovudine (HIVRTMC; Asp-67→Asn, Lys-70→Arg, Thr-215→Phe, Lys-219→Gln), whereas a variant with changes in the first three of these residues (HIVRTMC/F) had a partially resistant phenotype (13). This finding indicates that the highly resistant clinical isolates that we identified with these three specific mutations in RT (but wild type at codon 219) are likely to have an additional mutation (or mutations).

To gain further insight into the initial mutational events occurring during the development of resistance, we have attempted in this study to isolate variants in cell culture by passage of sensitive HIV in zidovudine. We also passaged partially resistant variants in zidovudine with the aim of selecting fully resistant virus which might have gained an additional mutation not previously recognized. We used the sensitive strain HXB2 plus two partially resistant variants (HIVRTMF and HIVRTMC/F), derived from HXB2 by site-directed mutagenesis (the RT mutations in these strains are shown in Table 1). The viruses were assessed for sensitivity changes after each passage by quantitative plaque reduction assay in HeLa CD4$^+$ cells (4, 11). We also used a polymerase chain reaction (PCR) procedure employing selective oligonucleotide primers, which enabled rapid genetic analysis of RT by discrimination of wild-type and mutant residues at each of the four codons of interest (13).

In this study, we were able to reproduce in vitro the initial order of appearance of resistance mutations seen in HIV clinical isolates (2). In addition, highly resistant virus selected by passage of the partially resistant variant HIVRTMC/F in zidovudine acquired a novel amino acid substitution at RT codon 219 that conferred the observed additional degree of resistance.

MATERIALS AND METHODS

Cells and virus. The human T-lymphoblastoid cell line MT-2 (9) was used to propagate HIV. These cells were

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TABLE 1. Genetic analysis of RT from HIV passaged in zidovudine[a]

Passage no.	HXB2					HIVRTMF					HIVRTMC/F				
	Zidovudine concn (μM)	RT genotype				Zidovudine concn (μM)	RT genotype				Zidovudine concn (μM)	RT genotype			
		67	70	215	219		67	70	215	219		67	70	215	219
0		·	·	·	·		·	·	M	·		M	M	M	·
1	0.02	·	·	·	·	0.1	ND	ND	ND	ND	0.3	ND	ND	ND	ND
2	0.05	·	·	·	·	0.25	ND	ND	ND	ND	1	ND	ND	ND	ND
3	0.1	·	·	·	·	0.5	ND	ND	M	·	2	M	M	M	·
4	0.2	·	·	·	·	1	·	·	M	·	4	M	M	M	·
5	0.5	·	X	X	·	2.5	·	·	M	·	10	M	M	M	·
6	0.5	·	M	X	·	2.5	·	·	M	·	10	M	M	M	·
7	1	·	M	X	·	5	·	·	M	·	15	M	M	M	·
8	2	X	M	X	·	10	·	·	M	·	30	M	M	M	·
9	4	X	M	X	·	20	·	·	M	·	50	M	M	M	·
10	4	X	X	X	·	20	·	X	M	·	50	M	M	M	·
11	5	X	X	M	·	20	·	·	M	·	50	M	M	M	*
12	5	X	X	M	·	20	·	·	M	·	50	M	M	M	*

[a] After each passage, DNA was extracted from MT-2 cell pellets and analyzed by selective PCR to discriminate wild-type from mutant residues at RT codons 67, 70, 215, and 219. Notation: ·, wild type; M, mutant; X, mixture of wild type and mutant; ND, not determined. Analysis of HIVRTMC/F passage 11 and 12 isolates is presented (*) to indicate that either a weak or no PCR signal was obtained with the selective primer pairs used.

routinely maintained in RPM1 1640 medium containing 10% (vol/vol) fetal calf serum plus antibiotics (RPM1/10), and Polybrene (2 μg/ml) was included for growth of HIV. HT4-6C cells (HeLa cells expressing the human CD4 receptor) were used to determine sensitivity of HIV to zidovudine (4). This cell line was maintained in Dulbecco modified Eagle medium (DMEM) containing 10% (vol/vol) fetal calf serum plus antibiotics (DMEM10). The HIV strains used in this study were the wild-type virus HXB2, which was derived from the infectious molecular clone pHXB2-D (6), and two partially zidovudine resistant variants, HIVRTMF and HIVRTMC/F (13). These variants were constructed by site-directed mutagenesis of pHXB2-D with the following mutations in RT: Thr-215→Tyr (HIVRTMF); and Asp-67→Asn, Lys-70→Arg, and Thr-215→Tyr (HIVRTMC/F). All virus stocks were initially prepared by transfection of MT-2 cells with DNA clones (13, 15). HIV stocks were stored as cell-free culture supernatants at −70°C.

Passage of HIV in MT-2 cells. MT-2 cells (2×10^6) were infected with cell-free HIV derived from culture supernatants at low multiplicity (less than 0.1 50% tissue culture infective dose per cell, as determined by terminal dilution in MT-2 cells). Virus was allowed to adsorb for 1 h at 37°C at high cell density (4×10^6/ml) and then diluted into RPM1/10 medium (plus Polybrene) to a density of 4×10^5 cells per ml. At this point, zidovudine was added to the cultures initially (at passage 1) to give a concentration of about twice the 50% inhibitory dose (ID_{50}) value for each virus. Cultures were incubated at 37°C until a substantial cytopathic effect was observed (large numbers of giant cells), and then virus was recovered in the form of cell-free culture supernatants and stored in aliquots at −70°C. In addition, infected cells at each passage were pelleted at the time virus was harvested, washed once in cold phosphate-buffered saline (PBS), and stored as a pellet at −70°C for subsequent analysis of virus DNA. The concentration of zidovudine added to cultures at each passage was gradually increased as shown in Table 1.

Assessment of zidovudine sensitivity by plaque reduction assay. HIV sensitivity was assessed by reduction of plaques (foci of multinucleated giant cells) formed in monolayers of HT4-6C cells essentially as described previously (11). Cell-free virus obtained from MT-2 cell cultures was used to infect HT4-6C monolayers. Virus was allowed to adsorb for 1 h at 37°C, and then this inoculum was removed to eliminate any zidovudine carried over from MT-2 cell cultures. Medium was then added (DMEM containing 5% fetal calf serum plus antibiotics), and cultures were incubated at 37°C for 3 days before monolayers were fixed with 10% (vol/vol) formaldehyde (in PBS) and stained with 0.25% (wt/vol) methyl violet solution. ID_{50} values were evaluated from plaque numbers obtained at different zidovudine concentrations. HIV isolates were considered sensitive with ID_{50} values up to 0.05 μM, partially resistant with an ID_{50} greater than 0.05 μM and less than 1 μM, and highly resistant with an ID_{50} value of >1 μM.

Genetic analysis of RT by selective PCR. DNA was obtained from infected MT-2 cell pellets stored at −70°C by detergent lysis and proteinase K digestion, followed by phenol extraction as detailed elsewhere (13); 1 μg of this DNA was used directly in each PCR. Selective oligonucleotide primers (with variation at the 3′ position) were used in PCR assays paired with common primers, allowing discrimination of wild-type from specific mutant residues at codons 67, 70, 215, and 219 in RT (13). DNA products of each PCR were analyzed by electrophoresis in 1.5% agarose–Tris-borate-EDTA (TBE) gels or composite 3% NuSieve (FMC BioProducts)–1% agarose-TBE gels and visualized by staining with ethidium bromide (1 μg/ml).

The mutation to Glu at codon 219 was identified by using the selective primer 4M/E (5′-AGGTTCTTTCTGATGTTT TAC-3′) paired with the common primer B (5′-GGATGG AAAGGATCACC-3′). PCR conditions with these primers were identical to those described for primer pairs B+4W and B+4M to discriminate wild type (Lys) from mutant (Gln) at codon 219 (13). Oligonucleotides were synthesized on an Applied Biosystems 394 machine.

Cloning and nucleotide sequence analysis of RT. The RT coding region was amplified by PCR from MT-2 cell-infected DNA and inserted into the M13 vector mptac18.1 as described previously (13, 15). Clones expressing active RT in *Escherichia coli* were identified and then sequenced by the dideoxynucleotide chain termination method (20). Specific oligonucleotide primers were used to enable sequence analysis of selected regions of the RT (14).

Construction of HIV with a mutation at codon 219 (Lys→Glu) in RT. The M13 mutant clone RTMC/F (13) was used as the target for site-directed mutagenesis to introduce a change (Lys→Glu) at codon 219. This clone contains a 2.55-kb fragment of the HIV *pol* gene with three specific mutations introduced at three codons in RT (Asp-67→Asn, Lys-70→Arg, and Thr-215→Tyr). Mutagenesis was carried out by the method of Kunkel et al. (10), and a clone with the correct mutation (termed RTMC/F[219E]) was confirmed by nucleotide sequencing (20). Infectious HIV containing the indicated mutations was isolated by recombination of the *pol* gene fragment from RTMC/F[219E] and wild-type pHXB2-D having had most of the RT region deleted (13). DNA (5 μg of each clone) was mixed and used to transfect MT-2 cells by electroporation, using a Bio-Rad Gene Pulser as described previously (13, 15). Virus was obtained in the form of cell-free culture supernatant at the time maximum cytopathic effect was observed. The genotype of this mutant strain, termed HIVRTMC/F[219E], was verified by the selective PCR procedure with primer pair B+4M/E.

RESULTS

Passage of HIV in the presence of zidovudine. To assess the potential development of zidovudine resistance in vitro, HIV strains were passaged in MT-2 cells a total of 12 times with increasing amounts of the drug. Wild-type virus HXB2 and two partially resistant mutants (HIVRTMF and HIVRTMC/F) were used to infect cells at a multiplicity of 0.1 50% tissue culture infective dose per cell. The zidovudine concentration in the first passage was about twice the ID_{50} value of each virus. Subsequently, fresh MT-2 cells were infected with HIV from culture supernatants in the presence of gradually increasing amounts of zidovudine (Table 1). The amount of virus recovered in culture supernatants was determined by titration both in MT-2 cells and in the HeLa $CD4^+$ line, HT4-6C. In addition, pellets of infected cells were stored at −70°C for subsequent genetic analysis of virus DNA.

Sensitivity of HIV strains following exposure to zidovudine. Cell-free virus obtained during each passage series was assessed for zidovudine sensitivity by plaque reduction assay in HT4-6C cells. The ID_{50} values of these isolates from passages 0 to 12 are shown in Fig. 1. The sensitivity of wild-type HXB2 decreased about 14-fold between passages 4 to 8, with ID_{50} values increasing from 0.014 μM initially to 0.2 μM by passage 12. Similarly, the partially resistant variant HIVRTMC/F also decreased in sensitivity during exposure to zidovudine. This change was observed between passages 7 to 11, as the ID_{50} increased from around 0.4 to 2.5 μM. Therefore, highly resistant virus had been derived from HIVRTMC/F through selection pressure with zidovudine. By contrast, little change in sensitivity was seen with HIVRTMF after 12 passages in zidovudine, even though the concentration of drug was increased from 0.1 to 20 μM in the MT-2 cell cultures (Table 1).

Genetic analysis of virus recovered by passage in zidovudine. To determine any genetic changes occurring during passage of HIV in the presence of zidovudine, we analyzed the RT of isolates at every passage by examination of DNA from infected MT-2 cells. This was achieved by using a selective PCR procedure designed to discriminate mutant from wild-type residues at codons 67, 70, 215, and 219 in RT (13). As expected from the sensitivity data shown in Fig. 1, no additional mutations were seen in HIVRTMF isolates from passages 1 to 12 (Table 1). By contrast, mutations were

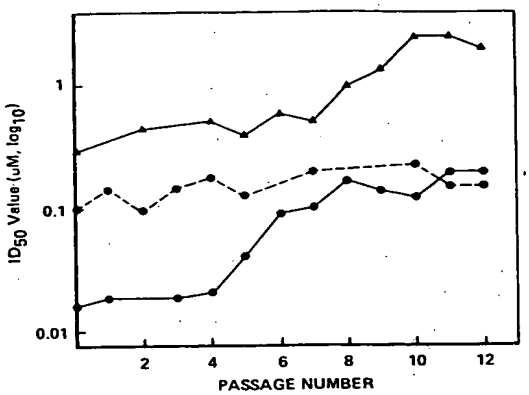

FIG. 1. Sensitivity of HIV strains passaged in MT-2 cells in the presence of zidovudine. HIV strains HXB2 (●–●), HIVRTMF (●----●), and HIVRTMC/F (▲) were used to infect MT-2 cells, which were then cultured with zidovudine. Virus recovered from culture supernatants from each infection was then serially passaged in the presence of increasing concentrations of zidovudine (shown in Table 1). Virus from each passage was titrated in HT4-6C cells, and the sensitivity of these isolates was determined by plaque reduction assay in this cell line. Sensitivity (ID_{50}) values were derived and are shown plotted against the passage number for each virus strain.

evident in HXB2 RT by passages 5 to 6, which was around the point when a change in sensitivity was first observed. The first codon appearing fully mutant was 70, which changed to a mixture by passage 11, when codon 215 became fully mutant (Table 1). There was also evidence of mutation at codon 67 by passage 8, when a mixed population was seen. With the variant HIVRTMC/F, we were particularly interested in determining whether a mutation occurred at codon 219. However, no change was apparent until passage 11, when codon 219 seemed to alter since only a weak or no PCR signal was detected (Table 1 and Fig. 2).

To analyze further the mutations detected by selective PCR, the RT coding region was amplified by PCR from DNA obtained from cells infected with passage 12 HXB2 and

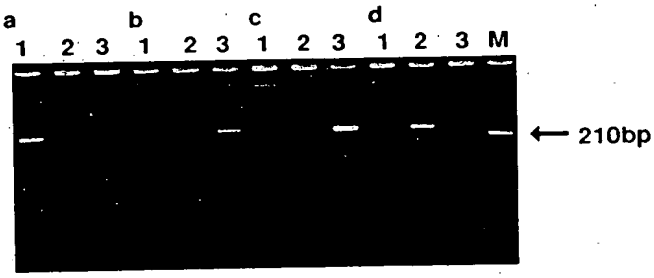

FIG. 2. Selective PCR analysis of HIV RT codon 219. DNA extracted from MT-2 cells infected with HIV isolates HIVRTMC/Fp0 (a), HIVRTMC/Fp12 (b), HIVRTMC/F[219E] (c), and HIVRTMC[219Q] (d) was subjected to PCR analysis using primers selective for specific nucleotides encoding different amino acids at RT codon 219. Primer pair B+4W was used to identify wild-type Lys-219 (lanes 1), primer pair B+4M was used to identify a mutant Gln-219 residue (lanes 2), and primer pair B+4M/E was used to identify a mutant Glu-219 residue (lanes 3). PCR products are shown separated on a 3% NuSieve–1% agarose-TBE gel and stained with ethidium bromide. The expected size of the specific PCR products from these primer pairs was 222 bp.

TABLE 2. Genetic and biological analysis of passage 12 isolates and a variant created by site-directed mutagenesis

Isolate	Zidovudine ID_{50} (μM)[a]	RT sequence[b]
HXB2	0.014	Asp-67 Lys-70 Thr-215 Lys-219
HXB2p12	0.2	Asp-67 Lys-70 Tyr-215 Lys-219
HIVRTMC/F	0.2	Asn-67 Arg-70 Tyr-215 Lys-219
HIVRTMC/Fp12	2	Asn-67 Arg-70 Tyr-215 Glu-219
HIVRTMC/F[219E]	4.5	Asn-67 Arg-70 Tyr-215 Glu-219
HIVRTMC	2	Asn-67 Arg-70 Phe-215 Gln-219

[a] Determined by plaque reduction in HT4-6C cells

[b] The RT coding region from each virus (or the M13 clone used to create a variant by mutagenesis) was subjected to nucleotide sequence analysis in the areas where resistance mutations had previously been identified. Strain HIVRTMC/F[219E] was derived from HIVRTMC/F by site-directed mutagenesis of codon 219 (Lys→Glu) in the RT.

HIVRTMC/F. These 1.7-kb fragments were cloned into the M13 vector mptac18.1 (15), and selected regions were sequenced. This analysis revealed a double-nucleotide change at codon 215 (Thr→Tyr) in HXB2p12 (Table 2), which was identical to a common mutation observed in clinical isolates after zidovudine treatment (14). The mixed population identified by selective PCR at codons 67 and 70 was not revealed by sequencing M13 RT clones from HXB2p12. This was almost certainly because too few clones (in this case four) were analyzed to give reliable information about mixtures of genotypes from this isolate. Nucleotide sequence analysis of RT clones from passage 12 HIVRTMC/F (HIVRTMC/Fp12) revealed a single change at codon 219 (Lys→Glu). However, this was different from the mutation (Lys→Gln) seen in clinical isolates at this residue (14).

Construction by mutagenesis of HIVRTMC/F with Lys→Glu at RT codon 219. It was likely that the mutation at codon 219 (Lys→Glu) seen in isolate HIVRTMC/Fp12 conferred increased resistance on this virus. However, to prove that this was the case, we decided to construct a virus based on HIVRTMC/F with this additional mutation in RT. Therefore, the mutation A to G (AAA; Lys→GAA, Glu) was created in M13 clone RTMC/F by site-directed mutagenesis. Infectious virus was recovered by cotransfection of this clone with the pHXB2-D provirus missing most of the RT region (see Materials and Methods). Virus obtained from this culture (HIVRTMC/F[219E]) was assessed for zidovudine sensitivity by plaque reduction assay in HT4-6C cells (Table 2). Introduction of this mutation at codon 219 resulted in a considerable increase in resistance of the virus (ID_{50} value increased to 4.5 μM). This was similar to the increase observed with isolate HWRTMC/Fp12 recovered by passage in zidovudine and was also similar to the sensitivity of isolate HIVRTMC, which was created previously by mutagenesis with Lys219→Gln (Table 2).

Application of a selective PCR assay to identify HIV with Glu-219 in RT. In considering the possibility of the Lys-219→Glu mutation occurring in clinical isolates during treatment, we decided to establish an additional selective PCR assay to identify this change. PCR primer 4M/E was synthesized with the 3′ nucleotide complementary to the G residue in mutant codon 219 (GAA). In addition, a deliberate A/A mismatch was designed into this primer at the residue next to the 3′ nucleotide to generate greater discrimination of the mutation (13). Results of the use of primer 4M/E paired with common primer B are shown in Fig. 2. DNA was extracted from MT-2 cells infected with virus isolates, including HIVRTMC/Fp12 and HIVRTMC/F[219E], and used in selective PCR whereby primer B was paired separately with either primer 4W, 4M, or 4M/E. PCR products were run on an agarose gel and stained with ethidium bromide (Fig. 2). A DNA band of the correct size appeared only with primer pair B+4M/E on analysis of isolates HIVRTMC/Fp12 and HIVRTMC/F[219E]. Isolate HIVRTMC/Fp0 (before zidovudine passage) was wild type (Lys-219), and the fully resistant mutant HIVRTMC gave a specific PCR band only with primer pair B+4M (indicating Gln-219).

DISCUSSION

The isolation of zidovudine-resistant HIV from individuals during therapy is now well documented (3, 12, 18). Characterization of these virus isolates has allowed cross-resistance profiles to be determined and the major mutations in RT conferring resistance to be identified (11, 14). Despite these advances, resistance studies might also be facilitated if systems were available to select HIV mutants in cell culture. Unfortunately, it has so far proved difficult to obtain such HIV strains by passage in zidovudine (15, 21). The reason for this is unclear, especially as the selection of zidovudine-resistant feline immunodeficiency virus in culture has been reported recently (17). However, we have now demonstrated the successful selection of HIV strains with reduced zidovudine sensitivity by exposure of virus to inhibitor in cell culture.

When we initiated the experiments reported here, we anticipated that it might be possible to induce increased resistance only in virus strains already partially resistant to the drug. The wild-type strain HXB2 was passaged in parallel to partially resistant mutants, although on the basis of results of previous studies we did not expect to observe a sensitivity change with this virus (21; unpublished results). The fact that we were able to identify HXB2 with altered sensitivity in addition to the partially resistant mutant HIVRTMC/F was almost certainly because we could take advantage of assay systems developed to investigate clinical isolates. Thus, the HT4-6C cell plaque reduction assay enabled recognition of relatively subtle sensitivity changes in passaged virus that would have been difficult to identify in less quantitative assays based on infection of T cells (1, 7, 8, 11, 16, 19). In addition, our selective PCR procedure allowed us to analyze those specific residues in RT known to modulate zidovudine sensitivity.

It is interesting to compare the pattern of resistance development in vitro with the situation observed with HIV isolates from treated individuals (2). Analysis of sequential isolates from patients initially asymptomatic has demonstrated that mutation at codon 70 in RT commonly occurs early, although this consistently seems to disappear later during therapy, being replaced by a stable mutation at codon 215 (2). At this stage, isolated virus tested in vitro is partially resistant to zidovudine (2, 3). This pattern is similar to that observed in this study during passage of wild-type HIV in culture, as codon 70 became fully mutant in RT before a fully mutant population was observed at codon 215, although by passage 10 codon 70 appeared to revert to a mixture (Table 1). It is not clear at present whether true genetic reversion occurs at codon 70 either in clinical isolates or in culture passaged virus. Alternatively, it could be that shifts in virus populations occur, perhaps because HIV with a codon 215 mutation has some growth advantage over isolates with a single mutation at codon 70. However, it is worth pointing

out that we have not observed a significant difference in the growth of these variants in cell culture (13). Experiments are under way to examine single biological and genetic clones from mixed populations to gain further insight into the mutational events occurring at these times.

In contrast to our observations with passaged wild-type HXB2, in which the mutation identified in RT at codon 215 (Thr→Tyr) is commonly seen in clinical isolates following zidovudine treatment, genetic analysis of the partially resistant strain HIVRTMC/F revealed a novel mutation in RT following zidovudine passage. This mutation at codon 219 was Lys→Glu in the HIVRTMC/F variant selected in cell culture, whereas the substitution seen at codon 219 in clinical isolates was Lys→Gln (14). We used site-directed mutagenesis to demonstrate that the Lys-219→Glu mutation in HIVRTMC/F was sufficient to change the virus from partially to highly resistant. In view of the possibility that this mutation might occur in clinical isolates, we adapted the selective PCR procedure to enable screening for such a change in clinical material. It is interesting to speculate on why the variant HIVRTMF, with a Thr→Tyr substitution at codon 215, did not alter significantly in sensitivity during passage in zidovudine. It may have been that the zidovudine concentration used was insufficient to exert pressure to select mutations. Alternatively, the apparent interplay between mutations at codons 70 and 215 during resistance development might require mutations at codon 70 to occur first in order to drive additional mutations.

In summary, we have been able to select HIV variants with increased resistance to zidovudine by passage in cell culture. Wild-type virus behaved similarly to clinical isolates with respect to the nature of mutations in RT and their order of appearance. A partially resistant strain became highly resistant upon passage in zidovudine, but this variant had a novel mutation at codon 219 in RT. It seems likely that another novel mutation(s) in RT has yet to be recognized, although care will be required to prove that any additional mutations seen in the RT of clinical isolates modulate zidovudine resistance. To rule out natural variation, it is essential to carry out site-directed mutagenesis of infectious molecular clones and careful sensitivity analysis of rescued virus. Finally, the passage procedure described here could be applied to investigate the potential development of resistance to other inhibitors of HIV, such as 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine. However, it may turn out, as was the case with zidovudine, that the study of clinical isolates from individuals receiving these drugs will give the most rapid insights into sensitivity changes.

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